

Aus dem Department of Pathobiological Sciences
School of Veterinary Medicine
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und
Institut für Virologie der Universität Zürich
(Direktor: Prof. Dr. R. Wyler)

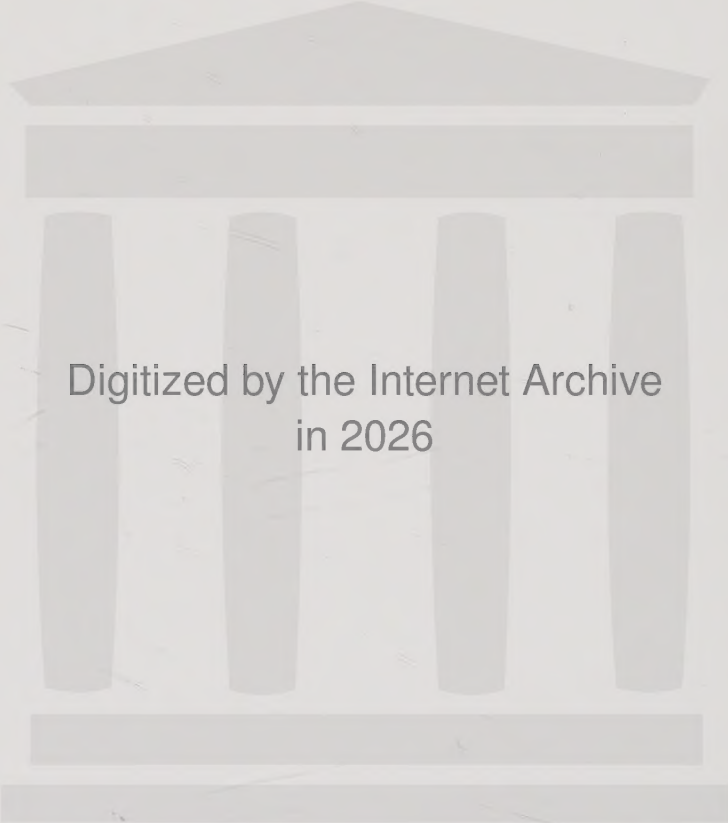
IDENTIFICATION OF SHARED EPITOPES BETWEEN
BLUETONGUE VIRUS SEROTYPES:
ANALYSIS WITH MONOCLONAL ANTIBODIES

Inaugural-Dissertation
zur Erlangung der Doktorwürde
der Veterinär-Medizinischen Fakultät
der Universität Zürich

vorgelegt von
Franziska B. Grieder
Tierärztin
von
Gelterkinden

Genehmigt auf Antrag von
Prof. Dr. R. Wyler, Referent
Prof. Dr. K. Schultz, Korreferent

Zürich 1987



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Meinen Eltern
sowie
meiner Tante und meinem Onkel
in Zuerich
gewidmet

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Bluetongue (BT) is an acute, infectious, non-contagious disease in both wild and domestic ruminants with a worldwide distribution. The disease is caused by Bluetongue virus (BTV), an Orbivirus, which is transmitted by an insect vector. In the USA, BT was first described as "Sore Muzzle in Sheep" in Texas and California in the late 1940s and early 1950s (18, 35). Today, outbreaks have been recorded in 32 states, and 48 states reported seropositive animals (22). Isolates have been obtained from cattle, sheep, and wild ruminants (white tail deer, water buffalo), but the most severe clinical disease is observed in sheep. In infected sheep, morbidity is greater than 50 percent, and mortality is about 10 percent. Twenty-four different BTV serotypes have been described, 5 serotypes are present in the USA, and one of them, BTV serotype 17, is found only in the USA.

Disease

In sheep, the first signs of disease following infection include increased respiratory rate and hyperemia of the muzzle, lips, and ears. This can lead to a blueish coloration of the tongue and oral mucus membranes, the sign which gave the disease its name. About 2-4 days post infection animals show fevers of 40.5°C - 41.5°C , moderate leukopenia, and edematous swelling of the lips, muzzle, and oral mucosa. Excessive salivation and nasal discharge usually develop at the same time. Ulceration of the oral mucosa is another sign of

infection, and extensive oral lesions can cause anorexia and lead to poor, general condition of the infected animal. Complicating sequelae can include vomiting and aspiration pneumonia. Additionally, coronitis may cause infected animals to stand with an arched back, or remain recumbent. In contrast, BTV infection in adult cattle is generally subclinical.

Another aspect of BT disease is in utero infection. Infection during the early stages of gestation can cause malformation and/or abortion. Fetuses infected during the second or third trimester of gestation are born with hydrancephaly and porencephaly (45, 46).

The pathogenesis of the disease in both the adult and the fetus is not clear, but the virus seems to have a tissue tropism for epithelial and endothelial cells (31). This preference explains the distribution of lesions (hemorrhages caused by endothelial damage, hydrancephalus caused by infection of ependymal cells). This predilection for ependymal cells is similar to another member of the family Reoviridae, Reovirus type I. This Reovirus infects ependymal cells in neonatal mice which result in hydrocephalus (37, 60).

Virus

BTV, as mentioned earlier, belongs to the genus Orbivirus in the family Reoviridae (International Committee on Taxonomy of Viruses, ICTV). It has a diameter of about 65-80 nm (16, 67). The virus is an icosahedral particle with two protein coats, but no lipoprotein envelope. The inner protein shell is composed of 32 doughnut shaped units (capsomeres) which build the circular surface configuration. These ring-like structures are responsible for the name given to the whole genus (orbis means ring in latin). Within these shells are the 10 double-stranded (ds) segments of the ribonucleic acid (RNA) (15). They code for at least 7 structural proteins and 3 non-structural proteins, designated as P1-P10 (17). Replication takes place intracytoplasmatically; viroplasmal inclusions are regularly seen within the cytoplasm surrounding virus particles, and it is proposed that they are a byproduct of replication (34). BTV is grouped serologically within the Orbiviruses by complement fixation, immunofluorescence, or agar gel diffusion tests. Viral protein 7 (P7), the major core protein, is responsible for the group specificity. The 24 serotypes of BTV are determined by the reaction of the two viral surface proteins, viral proteins P2 and P5. The virus neutralization test is the standard method for serotyping, and P2 is known to play an important role in viral neutralization (17) and viral attachment (26). The role of P5 is not well established at this time. It is conceivable, however, that the second outer capsid protein has some function in viral attachment; if not in mammalian cells then possibly in the insect vector.

Vector

In North America, the insect vector of BTV is Culicoides variipennis, a small biting gnat or midge. Because of its nocturnal habits, the dipterous midge attacks its hosts on night pasture and feedlots. Recently, studies by Stott et al. (57) showed that transovarial transmission in another insect vector can occur. The argasid tick, Ornithodoros coriaceus, was laboratory infected with BTV 10 by feeding on infected chicken embryos or sheep blood. Transmission of BTV to susceptible sheep by the infected tick suggests that the tick may be a potential vector for this virus.

Significance

Bluetongue is important for two reasons: 1) The clinical signs of disease are very similar to signs seen in other viral diseases. In particular, there are considerable and possibly misleading similarities in clinical signs of BT disease with such highly contagious diseases like vesicular stomatitis, infectious bovine rhino tracheitis, or foot-and-mouth disease. 2) Economic impact in the sheep industry can be substantial. Losses in sheep are caused by prolonged incapacitation, fetal deformities, decrease in wool yields, depreciation of the affected premises, and death (31). Losses in the cattle industry are an additional economic factor. Restrictions in the livestock trade, including animal and semen export to the European Community (11) are of international importance. For these reasons research in the field of BT is important, and

further knowledge of the pathogenesis of the disease and the protective immune response of the host would be valuable for developing better diagnostic tests and vaccines.

Vaccines

An ideal BT vaccine would: 1) prevent infection with all regional BTV serotypes; 2) produce no clinical signs of disease in the inoculated animals or their fetuses; 3) have to be incapable of reversion to virulence or reassortment with wild type virus; 4) permit the discrimination of vaccinated from infected animals; and 5) have to be stable. The current BTV vaccines induce protective immunity. However, these vaccines can cause disease in the fetus, and they have the potential for genetic reassortment with a wild-type virus. This has been demonstrated in experiments by Collisson et al. (10) where wild type BTV 10 was compared to 2 vaccine strains of BTV 10 by oligonucleotide fingerprint. The results indicate that the field isolate was the result of naturally occurring reassortment of genome segments derived from different BTV strains and serotypes. Another problem with current used vaccines is that it is not possible to easily discriminate infected from vaccinated animals. Therefore, alternative approaches for vaccine production have been examined. A beta-propiolacton inactivated bivalent vaccine has been shown to induce a neutralizing AB response in sheep (48). Unfortunately, no challenge studies were conducted in these experiments. More recently, Stott et al. (56) inoculated sheep with a binary

ethylenimine inactivated virus preparation. This vaccine did not produce a neutralizing AB response. Interestingly, it did reduce the level of viremia in the challenged animals. However, some sheep inoculated with this product when challenged with virulent virus developed more serious disease than the control animals. The protection in the animals which showed reduced levels of viremia correlated with an in vitro antigen specific lymphocyte transformation assay. This suggests that cellular immunity may be important in protection from BTV disease.

It is not clear what contribution humoral versus cellular immunity has in protection from infection. However, the fact that protective immunity and neutralizing antibodies are basically serotype specific suggests that neutralizing antibodies play an important role in protection (32).

An additional problem in the vaccine production is the plurality of the BTV serotypes and the serotype restricted protective immune response, particularly in areas where up to 15 different serotypes of BTV are present each year (e.g. South Africa). Polyvalent vaccines composed of up to 15 serotypes of BTV are used to immunize sheep in South Africa. However, this may not be necessary. It was demonstrated that sheep previously exposed to BTV serotypes 3 and 4 were resistant to infection with serotype 6 (27), and additionally that these sheep produced neutralizing ABs to a large number of other BTV serotypes. This finding indicates that cross-reactive,

protective immunity following exposure to a limited number of BTV serotypes can occur. The phenomenon of cross-reactivity in vivo is called heterotypic immunity (1). The mechanism for heterotypic immunity in BT disease is not known, but one explanation involves shared or conserved epitopes between different serotypes. The detection and characterization of such shared epitopes, which are involved in virus neutralization, would be of great help for future vaccine development where multiple serotypes are present in a particular geographical region.

The purpose of this investigation was to test the hypothesis that there are shared neutralizing epitopes among different BTV serotypes.

PRODUCTION AND CHARACTERIZATION OF
NEUTRALIZING MONOCLONAL ANTIBODIES
AGAINST BLUETONGUE VIRUS 17

ABSTRACT

Thirteen monoclonal antibodies with in vitro BTV 17 neutralizing activity were produced by standard fusion technique. The relative activity of each of the monoclonal antibodies to neutralize BTV 17 was determined in a virus neutralization assay. The epitopes involved in neutralization are located on the viral protein P2, a 98 kilodalton protein, as demonstrated by radioimmunoprecipitations with the different neutralizing monoclonal antibodies. P2 is the major outer capsid protein of the Bluetongue virus. These results are in agreement with previously published observations that P2 is the protein involved in neutralization (2, 38). Two spatially separate and different antigenic determinants or epitopes were defined on P2. This was demonstrated by competitive binding assays in which two different neutralizing monoclonal antibodies simultaneously bound to the virus adsorbed on target cells.

The characterization of some of the biological functions of the monoclonal antibodies is discussed.

PRODUCTION AND CHARACTERIZATION OF NEUTRALIZING MONOCLONAL
ANTIBODIES TO BLUETONGUE VIRUS 17

Monoclonal antibodies are the fusion product of spleen cells of mice, previously immunized with an antigen, and a myeloma cell line, which grows in tissue culture (39, 49). The fusion is performed in the presence of polyethylene glycol (PEG). A successful lymphocyte-myeloma fusion provides a tool to answer questions in immunology and biology, because monoclonal antibodies unite the advantages of being 1) homogeneous or specific for a single antigenic determinant, 2) producible in a reasonably high titer, and 3) in principle, unlimited in supply. Neutralizing MABs against BTV 17 provide a useful reagent to further characterize the mechanism of neutralization and therefore the genesis of protective immunity against this virus. To characterize the biological function and activity of neutralizing MABs, used to map epitopes on different serotypes, we specifically wanted to determine:

1. if different clones of MABs would exhibit different neutralizing activities,
2. if neutralizing MABs were specific for one viral protein, and
3. if neutralizing MABs would bind the same epitope on the viral protein involved in neutralization.

MATERIALS AND METHODS

Animals. Four male and 12 female Balb/c Bj mice (obtained from Jackson labs, Bar Harbor, ME) kept in an isolated, closed colony were bred at 4 week intervals to produce the mice used in all the experiments. The final colony size was about 100 mice.

Virus and Cells. MDBK cells (Madin-Darby-Bovine-Kidney), a bovine fibroblast cell line, were used for all the experiments. The cells were grown in sterile filtered Eagle's minimum essential medium (MEM) (Gibco Laboratories, Grand Island, NY), supplemented with 8 percent heat inactivated fetal bovine serum (FBS).

Bluetongue virus serotype 17 (BTV 17) was a gift from Dr. R. Schultz, Dept. Pathobiological Sciences, University of Wisconsin, Madison. The virus was plaque cloned three times to avoid contamination with defective interfering particles. Briefly, serial 10-fold dilutions of BTV were incubated on confluent monolayers of MDBK cells grown in 6-well plates. After 1 hour at 37°C the viral suspension was decanted and the monolayers were covered with 3.0 ml of 0.5 percent agarose (Miles type LS, Miles Scientific Laboratories, Inc., Naperville, IL) in MEM at 44°C. The plates were carefully swirled to allow the agarose to mix with the remaining media. During an incubation of 48 hours at 37°C the virus was allowed to multiply, and after this time plaques were easily visible through the agarose overlayer. Using a sterile Pasteur

pipette, isolated plaques were picked and suspended in 2.0 ml of media without FBS. The single plaque of virus was then expanded using MDBK cells grown in a 75 T flask (Falcon, Becton Dickinson, Oxnard, CA). This process of plaque cloning was repeated 3 times. The virus harvested from the lysed cells was frozen at -70°C in media containing FBS, and will be referred to as stock virus. Stock virus stored under these conditions was stable up to a period of at least 1.5 years.

For use in some of the experiments, stock virus was partially purified by Huebschle's (23) modification of the procedure of Verwoerd (65). Confluent MDBK cell monolayers were infected with stock virus at a multiplicity of infection (MOI) of 1-5 for 12-16 hours. At this stage of infection, the MDBK cells were just starting to show cytopathic effects (CPE). After two washing steps using 7 ml trypsin diluent (phosphate buffered saline without magnesium and calcium, supplemented with 2 percent phenol red), the cell monolayer was enzymatically dispersed with 3 ml of 1 percent trypsin EDTA, and the cells were suspended in media containing FBS. For the remaining procedures the cell and virus suspension was kept at 4°C . The cells were pelleted and lysed with Tris buffer (0.002M Tris-HCl, pH 8.8). The lysis was repeated and the lysates were pooled. Virus was separated from the cellular fraction by five freon (trichlorotrifluoroethane, Aldrich Chemicals, Inc., Milwaukee, WI) extractions, each utilizing five times the volume of the aqueous fraction. The aqueous phase of the pooled extractions was then layered onto a 30 percent

sucrose cushion containing 0.02 percent (volume/volume) Tween 80 (Sigma Chemical Company, St. Louis, MO) and pelleted for 4.8 hours at 100,000 x g (Beckman SW 28 rotor, Beckman Instruments, Arlington Heights, IL). The sucrose cushion pellet was then resuspended in 0.002 Tris-HCl and repelleted either under the same conditions as before, or for 15 hours at 32,000 x g. The virus was then resuspended in 0.002M Tris-HCl pH 8.8 containing 0.5 percent bovine serum albumin (BSA) and stored at -70°C.

The number of plaque forming units (PFUs) in a virus suspension was determined by a serial 10-fold titration. The procedure used was essentially identical to the one described under plaque cloning. After the 48 hours incubation under agarose, however, the monolayers were stained and fixed with crystal violet (EM Science Industries, Inc., Gibbstown, NJ) and formalin for several hours. The agarose cover was removed, plaques were counted by examination under an inverted microscope in wells that had between 30-300 plaques and the number of PFUs per ml of the original suspension was calculated.

Hybridoma Production. To obtain a mouse adapted Bluetongue virus, plaque cloned BTV 17 was grown in brains of Balb/c suckling mice. Neonatal mice less than 24 hours of age were inoculated intracranially with 10^2 - 10^3 PFUs of semipurified BTV 17. The animals were sacrificed when they started to show clinical signs of infection. The signs included discontinuation of nursing, subcutaneous hemorrhages

on head and back, hyperactivity, and occasionally head tremors. The brains were removed aseptically and macerated in physiological saline solution to give a 20 percent brain suspension. This brain suspension was mixed (50 percent volume/volume) with Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY) and administered as a priming dose intraperitoneally (250 μ l) to adult Balb/c mice. A second immunization 14 days later followed the same protocol except that incomplete Freund's adjuvant was used. After another 14 days, purified virus was administered intravenously without adjuvants. Three days after the final immunization, the mice were anesthetized with methoxyflurane (Metofane^R, Pitman-Moore, Inc., Washington Crossing, NJ) and splenectomized. Spleen cells were harvested, washed in Dulbecco's minimum essential medium (DMEM) (Gibco Laboratories, Grand Island, NY), and fused with the NS-1 myeloma cell line (kindly supplied by Dr. R. Grieve, Dept. Pathobiol. Sci., University of Wisconsin, Madison). The method of Fazekas de St. Groth (13) for fusion of mouse lymphocytes with myeloma cells was followed, using polyethylene glycol (PEG 4000, EM Science, Darmstadt, West-Germany) in phosphate buffered saline (PBS) and Dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO). Cells were fused at a ratio of 10 lymphocytes to 1 myeloma cell. Hybridomas were grown and selected in 96-well-tissue culture plates (Corning Laboratory Products, Corning, NY) in DMEM supplemented with hypoxanthine-aminopterin-thymidine (HAT) (Sigma Chemical Company, St. Louis, MO), 2mM L-glutamine, and 20 percent FBS. Hybridoma colonies were tested for neutralizing

antibody secretion in a virus neutralization (VN) assay, and positive clones were transferred to 24-well-tissue culture plates. Hybridomas were recloned by limiting dilution at least three times and until all subclones showed the same subtype of immunoglobulin class. Cloned hybridomas were subsequently grown in DMEM containing 8 percent FBS in 25 T and 75 T flasks (Falcon, Becton Dickinson, Oxnard, CA) and culture supernatant was harvested.

Hybridomas and their antibodies were numbered according to the fusion and the particular well in which they were produced, i.e. the first number indicating the fusion, the second the particular plate in this fusion, and the third and fourth describe the well. The last part of the MAB name was determined by the number of the subcloning cycles of each well of the original hybridoma (see table 1).

Determination of Immunoglobulin Heavy Chain Class. All hybridoma clones were typed to determine the heavy chain class using an Ouchterlony agar gel diffusion screening kit (ICN Immuno Biologicals, Lisle, IL).

Neutralization Test. The virus neutralization test for screening hybridomas was performed in 96-well-tissue culture plates. Fifty μ l of hybridoma supernatant was mixed with 50 μ l of infected MDBK cell supernatant containing 5,000 PFUs of BTV 17. The virus-hybridoma mixture was incubated at 4°C for 1 hour. One hundred μ l of MEM with 8 percent FBS containing

50,000 MDBK cells was then added to each well and incubated at 37°C in 5 percent humidified CO₂ for 4 hours to allow the cells to adhere. The supernatant was then decanted and the monolayers were covered with 0.5 percent agarose in MEM. After 48 hours of incubation at 37°C the cells were fixed and stained as described for the virus titration, and examined for viral growth. Controls included 1. a viral back-titration to verify the correct virus titer, 2. normal uninfected cells to ensure the growth of the cells under the experimental conditions, 3. hybridoma supernatant as supplement to MDBK cells to demonstrate the non-toxicity of supernatant for the target cells, 4. a known negative hybridoma supernatant grown under the same conditions as the anti-BTV 17 clones incubated with virus to show the non-neutralizing effect of a nonsense supernatant, and 5. a known positive, anti-BTV 17 neutralizing ascites fluid (kindly provided by Dr. G. Letchworth, Dept. Vet. Sci., University of Wisconsin, Madison) to demonstrate positive neutralization.

The neutralizing ability of the MABs was graded as follows: Complete neutralization, i.e. no plaque formation in the cell monolayer after inoculation of 5,000 PFUs of BTV 17, were called strong neutralizer. Monoclonal antibodies which had neutralizing activity as compared to the negative control, but failed to protect the cell monolayer completely were called weak neutralizer. Monoclonal antibodies which failed to protect the cell monolayer from complete lyses by the inoculated virus were called negative neutralizer.

Monoclonal Antibody Purification. Monoclonal antibodies were purified using affinity columns specific for mouse immunoglobulins (Ig). The temperature during the procedure was kept at 4°C. Disposable columns (Biorad Laboratories, Richmond, CA) were packed with 2ml of either anti-mouse IgM-agarose (antibodies developed in goats suspended in 0.5 M NaCl containing 0.01 percent thimerosal) or protein A-sepharose (type CL-4B) (both from Sigma Chemical Company, St. Louis, MO). The columns were washed extensively with PBS prior to use. Hybridoma culture supernatant was then applied to the affinity column at a rate of 1ml/2min. After the whole volume of supernatant had passed through the column, the beads were washed with PBS using 30 times the bead volume. Antibody was eluted with 10 ml of 0.5 M acetic acid pH 2.4, containing 0.15 M NaCl. One half ml fractions of the eluate were collected in equal volumes of 0.2 M Tris-base, pH 10.8, and the pH of all samples was tested immediately and adjusted to neutral. Following complete elution of the MABs, the columns were regenerated with 0.2 M Tris-HCl, pH 7.0, containing 0.9 percent NaCl. The protein concentration in the different fractions was measured spectrophotometrically (Beckman DU-6 spectrophotometer, Beckman Instruments, Arlington Heights, IL) by comparing optical density readings with a standard curve made with BSA. These purified antibody fractions were subsequently tested for their neutralizing activity in a VN assay and assayed by polyacrylamide gel electrophoresis to detect possible contaminants.

Radiolabeling of BTV 17 Infected MDBK Cells. Confluent monolayers of MDBK cells grown in 75 T flasks were infected with plaque cloned BTV 17 using a MOI of 3-5. Ten hours post infection, the culture supernatant was removed, monolayers were washed twice with methionine free DMEM and incubated for 30 minutes with this same media. Monolayers were then incubated with ^{35}S -methionine containing DMEM for a subsequent 2 hour period. The radioactive supernatant was decanted and the cells were incubated with DMEM with L-methionine for 30 min at 37°C . The cells were lysed with 3 ml of NET-buffer (0.15 M NaCl, 0.04 M EDTA, 0.04 M Tris, 2 mM phenylmethylsulfonyl fluoride, pH 7.0), containing 0.03 percent NP-40 and 0.1 percent BSA, and with a final concentration of 0.1 percent Na-Deoxycholate. To remove cellular debris, the lysate was centrifuged at $100,000 \times g$ at 4°C for 30 min (Beckman SW 60, Beckman Instruments, Arlington Heights, IL) and then preadsorbed to protein A-sepharose (SPA) beads (Sigma Chemical Company, St. Louis, MO) coated with rabbit anti-mouse antibody, to decrease non-specific binding of antigen to beads.

Radioimmunoprecipitations. The Radioimmunoprecipitations (RIPs) were produced by incubating rabbit anti-mouse Ig antibody with 50 ul of staphylococcus protein A bound beads over night at 4°C . After 3x washing with NET washing solution (0.15M NaCl, 0.005M EDTA, 0.05M Tris, 0.05% Triton X 100), 1 ml supernatant of one of the anti-BTV 17 neutralizing clones was incubated with the rabbit antibody bound beads at 4°C for

several hours. Following 3 washing steps with NET solution, 70-100 ul of radiolabeled cell lysate (approximately 500,000 counts per minute) was incubated for 2 hours and after another 3 washing steps, bead-AB-complexes were ready for electrophoresis. Immune complexes were disrupted by heating the beads in the presence of sodium dodecyl sulfate (SDS) (Sigma Chemical Company, St. Louis, MO) and 2-mercaptoethanol.

Electrophoresis. Reducing SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 1.5 mm thick slab gels, using a 10 percent polyacrylamide (Sigma Chemical Company, St. Louis, MO) resolving gel and a 5 percent polyacrylamide stacking gel (36). The gels were run at constant current in a SDS-Tris-glycine buffer system at 10°C. Molecular weight estimation were calculated by comparison to the migration of 30ul of a standard molecular weight marker mixture at a protein concentration of 3mg/ml (Sigma Chemical Company, St. Louis, MO). The gels were stained and fixed in 0.1 percent Comassisse Brilliant Blue (Bethesda Research Laboratories, Gaithersburg, MD) and 10 percent trichloroacetic acid (TCA) for 1 hour at room temperature, and diffusion destained in 10 percent glacial acetic acid, and 20-40 percent methanol. After fluorographing in Enhance^R (du Pont, NEN Research Products, Boston, MA) for 45 min, and drying under vacuum, the gels were exposed to radiograph film (Kodak X-Omat AR, Kodak Company, Rochester, NY) at -70°C for a period of 3-7 days.

Competition Assay. MDBK cells were grown to approximately 90 percent confluency and then trypsinized from the flask surface, washed with PBS and counted. The experiment from this point was performed at 4°C. Five thousand cells and 100 infectious virus particles per cell of semipurified BTV 17 were combined and incubated for 1 hour. After 3 washings with PBS, in a first step, 1 ml of hybridoma culture supernatant was added to the cell-virus complex and incubated for 30 minutes. In the second step, culture supernatant from a different clone of a different Ig isotype was added to the complex and incubated for 30 minutes. Therefore, to determine if the second AB was present, in the third step, a fluorescein isothiocyanate (FITC) conjugated AB specific for mouse Ig of the second isotype (Sigma Chemical Company, St. Louis, MO) was incubated with the cell-virus-antibody complex for 30 min. Each of these steps was followed by 3 washings cycles with PBS. The cells were then fixed in 0.5 percent paraformaldehyde in PBS and analysed by flow cytometry (Epics-C, Coulter Electronics, Hialeah, FL). As a positive control, an aliquot of the cell-virus complex after incubation with the first MAB was stained with FITC conjugated AB specific for the isotype of the first MAB. Alternatively, as negative control, the cell-virus-complex was incubated only with the first MAB, but stained with the fluorescent AB specific for a different isotype.

RESULTS

Neutralizing Monoclonal Antibodies to BTV 17

A panel of 13 neutralizing MABs specific for BTV 17 was produced in two different fusions (table 1). There were 4 different isotypes produced, G₁, G_{2a}, G₃, and M. The neutralizing activity of the different monoclonal antibodies varied considerably. Fifty ul of hybridoma culture supernatant from three clones produced under similar conditions incubated on 50,000 MDBK cells completely neutralized 5,000 PFUs of BTV 17 (=strong neutralizer), whereas the same volume of other MABs reduced the number of plaques but failed to protect all the cells from lysis by the virus (=weak neutralizer) (table 2). The neutralizing activity of pooled supernatant from the same hybridomas harvested at different times gave the same results and justified the rating system employed even without knowledge of the actual number of Ig molecules.

Identification of the BTV 17 Viral Protein Responsible for Neutralization

The specificity of the MABs for viral proteins was determined by radioimmunoprecipitation (RIP). The identity and the molecular weight of the viral proteins were determined by comparison to known molecular weight standards run at the same gel and comparison to the protein precipitations with AB to known BTV viral proteins (2, 17).

MONOCLONAL ANTIBODY	ISOTYPE
82F5-2B5	M
83B2-3A	G ₁
83B2-11A	M
83C8-B1C	G ₃
91E2-2L8	M
92B4-B2	G ₃
92E5-2A	G ₁
92F8-8N	M
93E8-8E	G _{2A}
93H5-1B	G _{2A}
94B5-10A	M
94G9-4B16	M
94G10-8A	M

Table 1. Isotypes of neutralizing anti-BTV 17 monoclonal antibodies.

MONOCLONAL ANTIBODY	NEUTRALIZING ACTIVITY AGAINST BTV 17
82F5-2BF	strong
83B2-3A	weak
83B2-IIA	weak
83C8-B1C	strong
91E2-2L8	weak
92B4-B2	weak
92E5-2A	weak
92F8-8N	weak
93H5-1B	weak
93E8-8E	weak
94B5-10A	weak
94G9-4B16	strong
94G10-8A	weak

Table 2. Neutralizing activity of BTV 17 neutralizing monoclonal antibodies. Strong neutralization is defined as complete protection of the cell monolayer when incubated with 5,000 PFUs of BTV 17; weak neutralization is defined as plaque reduction on the cell monolayer when incubated with 5,000 PFUs of BTV 17, but failure to protect completely.

Precipitations with mouse ascites fluid containing MAB specific for P2 of BTV 17 (clone 6C2A.4.2, lane 5, figure 1), MAB specific for P7 of BTV 17 (clone 7D5C.1, lane 6, figure 1), and MAB specific for P9 of BTV 17 (clone 8A3B.6, lane 7, figure 1) (provided by Drs. G. Letchworth and J. Appleton) resulted in protein bands of 98, 48, and 43 kilodalton (KD), respectively. Additionally, rabbit anti-BTV 17 typing serum precipitated at least 6 viral associated bands, P2 (98 KD), P5 (66 KD), P6 (52 KD), P7 (48 KD), P8 (48 KD), and P9 (43 KD) (lane 8, figure 1).

When precipitations with 83C8-B1C and 82F5-2B5 were performed, both MABs precipitated a 98 KD protein (lanes 2 and 4, figure 1), presumed to be P2. Additional proteins were precipitated but were assumed to be the result of non-specific binding, because they appeared at the same or higher intensity in the controls (lanes 1 and 3, figure 1, control precipitations of radiolabeled BTV 17 proteins with nonsense AB). Results with other clones are shown in figure 2. All the clones (with the exception of 93H5, lane 5) precipitated the 98 KD protein. This 98 KD protein was not precipitated with control ABs (figure 2, lanes 1 and 4, nonsense IgM and IgG, respectively). There were more non-specific bands in the autoradiograph of this gel (figure 2) than in the previous autoradiographs. Non-specific binding either to SPA or to the antibody per se is constant in both gels, but may be more apparent in the second gel because of the relatively weak signals given by P2.

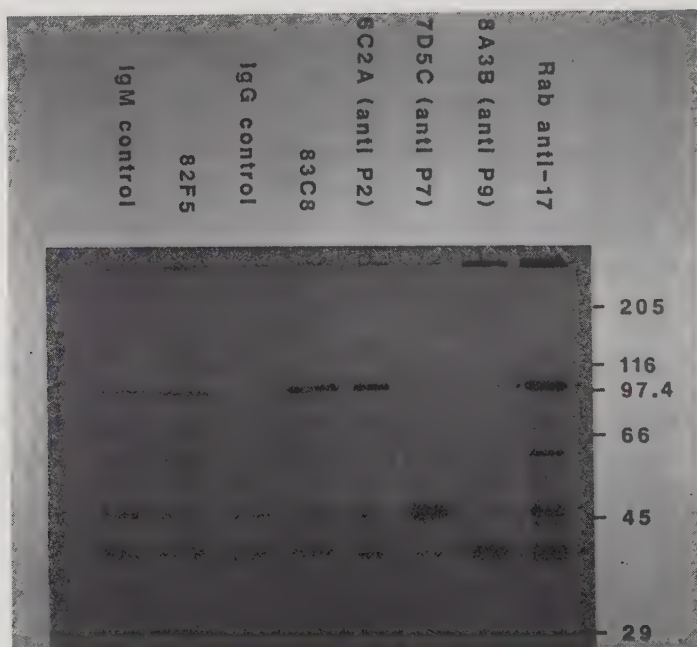


Figure 1. Autoradiograph of proteins immunoprecipitated by neutralizing anti-BTV 17 monoclonal antibodies (MAB). ^{35}S labeled BTV 17 proteins analysed on a 10% SDS-Polyacrylamide gel. Lanes 1 and 3, nonsense MAB; lane 2, neutralizing anti-BTV 17 MAB 82F5-2B5, an IgM; lane 4, neutralizing anti-BTV 17 MAB 83C8-B1C, an IgG; lane 5, anti-P2 BTV 17 MAB 6C2A; lane 6, anti-P7 BTV 17 MAB 7D5C; lane 7, anti-P9 BTV 17 MAB 8A3B; lane 8, rabbit anti-BTV 17 AB; molecular weights indicated at right side.

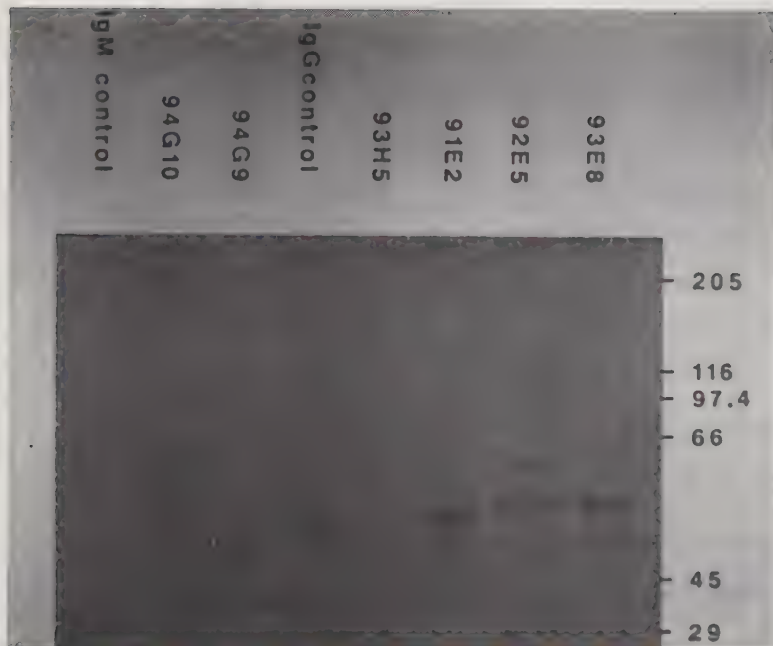


Figure 2. Autoradiograph of proteins immunoprecipitated by neutralizing anti-BTV 17 monoclonal antibodies (MAB). ³⁵S labeled BTV 17 proteins analysed on 10% SDS-Polyacrylamide gel. Lanes 1 and 4, nonsense MAB; lanes 2, 3, and 6, neutralizing anti-BTV 17 MABs, all IgM classes; lanes 5, 7, and 8, neutralizing anti-BTV 17 MABs, all IgG classes; molecular weights indicated at right side.

Identification of Different Epitopes on the BTV 17 Neutralizing Protein P2

Competition assays in combination with flow cytometric analysis (FACS) were performed to determine if the MABs bound to the same or to different epitopes on the neutralizing antigen of BTV 17. Whole infectious virus was adsorbed to MDBK cells. To prevent internalization of the virus, the temperature during the experiment was kept at 4°C. The two competing MABs were allowed to bind in sequence, i.e. AB 1 (IgM) was allowed to bind prior to the addition of AB 2 (IgG). The first MAB was added in saturating (excess) amount, and it was verified that this first MAB was bound to the cell-virus complex. The complexes were stained with fluorescein isothiocyanate (FITC) coupled AB specific for the isotype of the second MAB.

To establish the assay, virus adsorbed on MDBK cells were first incubated with one single neutralizing MAB, and subsequently stained with a FITC labeled AB specific for the isotype of that particular MAB. In figure 3, cell-virus complexes incubated with MAB 82F5-2B5, an IgM, were compared with cell-virus complexes alone (both test and control cells stained with FITC conjugated anti-IgM). The shift in green fluorescence of the cell-virus complexes incubated with neutralizing MABs 82F5-2B5 (histogram on the right, figure 3) indicates that 82F5-2B5 is bound to the cell-virus-complex.

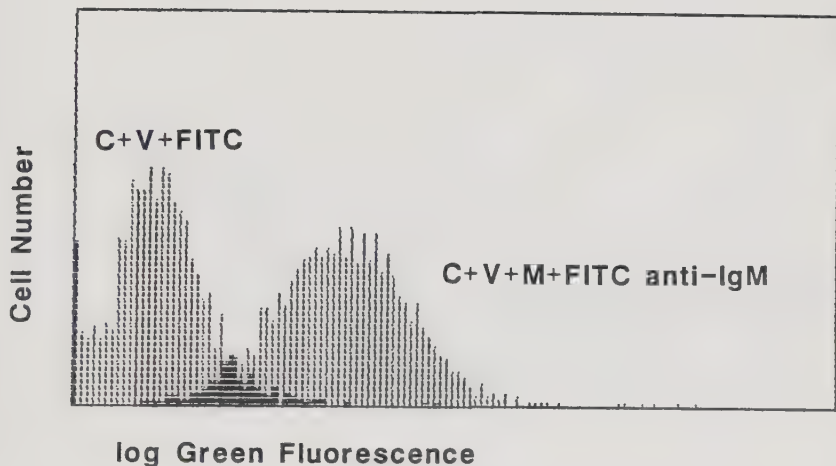


Figure 3. Relative fluorescence of cell-virus-complexes incubated with monoclonal antibody 82F5-2B5. MDBK cells (C); infectious Bluetongue virus 17 (V); neutralizing monoclonal antibody 82F5-2B5, isotype M (M); antibody specific for mouse IgM conjugated with FITC (FITC anti-IgM).

Using this technique, the competition assay was performed (see figure 4). The test sample, cells + virus + IgM + IgG + FITC conjugated anti-IgG AB, was compared to cells + virus + IgM + FITC conjugated anti-IgG AB. A positive and statistically significant shift in green fluorescence of the cell-virus-complexes incubated with two neutralizing MABs was observed. In figure 5, the test sample, cells + virus + IgM + IgG + FITC conjugated anti-IgG AB, was compared to cells + virus + IgM + FITC conjugated anti-IgM AB. The two graphs were superimposable and statistically not different. The analysis of the competition assay using the MABs in reverse sequence (incubation of the IgG MAB, 83C8-B1C, as first or blocking AB, and testing for subsequent binding of the IgM, 82F5-2B5, see figures 6-8) showed similar results as seen in figures 3-5.

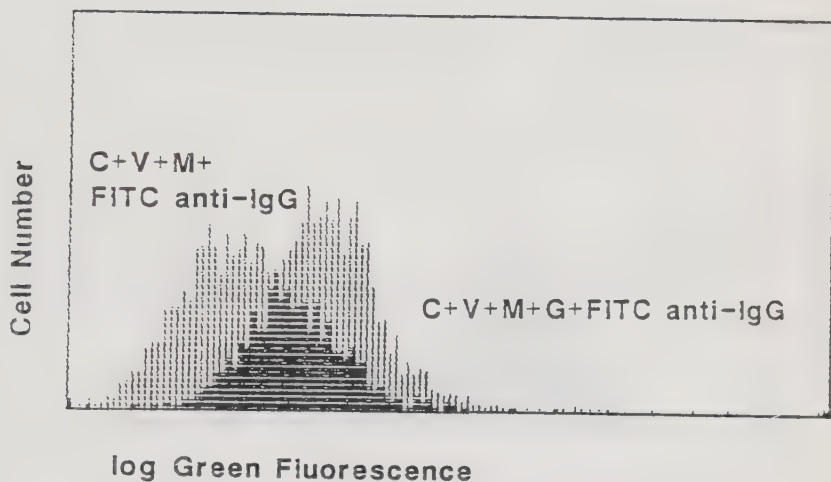


Figure 4. Relative fluorescence of a competition binding assay using two different neutralizing anti-BTV 17 monoclonal antibodies. Staining is specific for the second monoclonal antibody, 83C8-B1C, an IgG. MDBK cells (C); infectious Bluetongue virus 17 (V); neutralizing monoclonal antibody, 82F5-2B5, isotype M (M); neutralizing monoclonal antibody, 83C8-B1C, isotype G (G); antibody specific for mouse IgG conjugated with FITC (FITC anti-IgG).

Cell Number

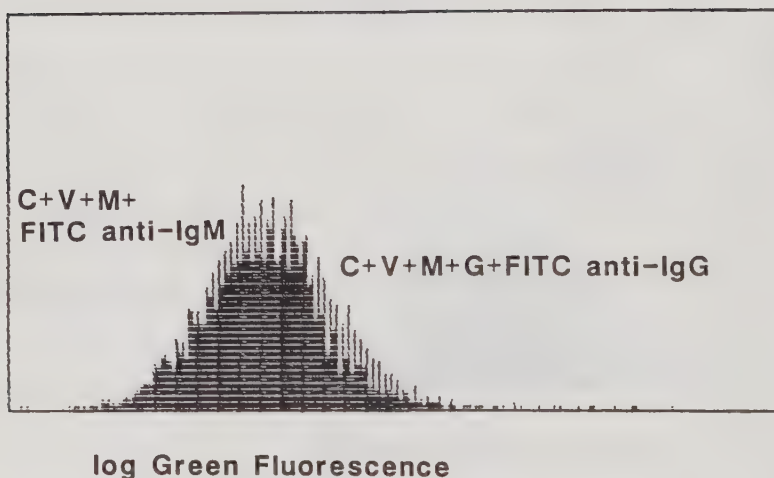


Figure 5. Relative fluorescence of a competition binding assay using two different neutralizing anti-BTV 17 monoclonal antibodies compared to cell-virus-complexes incubated with one monoclonal antibody. MDBK cells (C); infectious Bluetongue virus 17 (V); neutralizing monoclonal antibody, 82F5-2B5, isotype M (M); neutralizing monoclonal antibody, 83C8-B1C, isotype G (G); antibody specific for mouse IgM conjugated with FITC (FITC anti-IgM); antibody specific for mouse IgG conjugated with FITC (FITC anti-IgG).

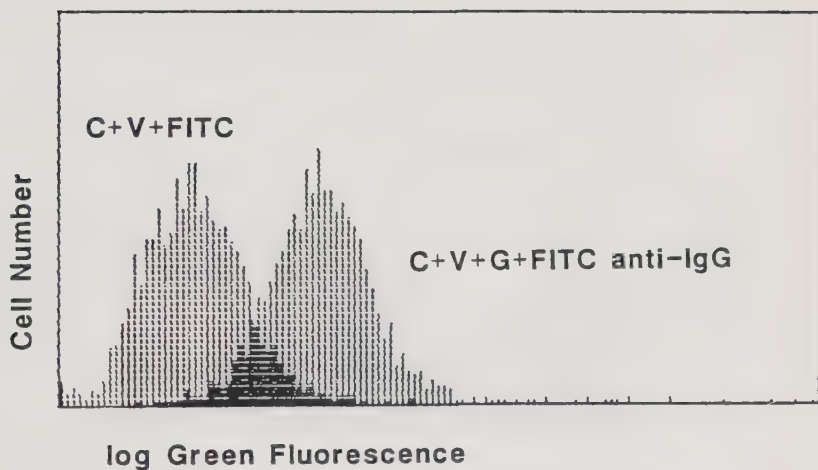


Figure 6. Relative fluorescence of cell-virus-complexes incubated with monoclonal antibody 83C8-B1C. MDBK cells (C); infectious Bluetongue virus 17 (V); neutralizing monoclonal antibody 83C8-B1C, isotype G (G); antibody specific for mouse IgG conjugated with FITC (FITC anti-IgG).

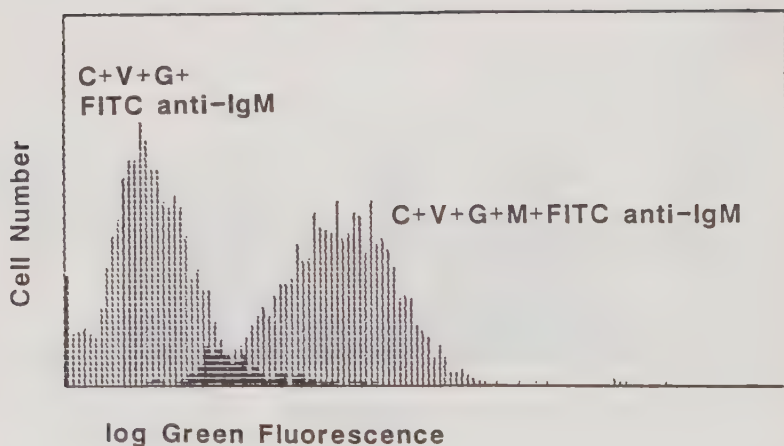


Figure 7. Relative fluorescence of a competition binding assay using two different neutralizing anti-BTV 17 monoclonal antibodies. Staining is specific for the second monoclonal antibody, 82F5-2B5, an IgM. MDBK cells (C); infectious Bluetongue virus 17 (V); neutralizing monoclonal antibody, 83C8-B1C, isotype G (G); neutralizing monoclonal antibody, 82F5-2B5, isotype M (M); antibody specific for mouse IgM conjugated with FITC (FITC anti-IgM).

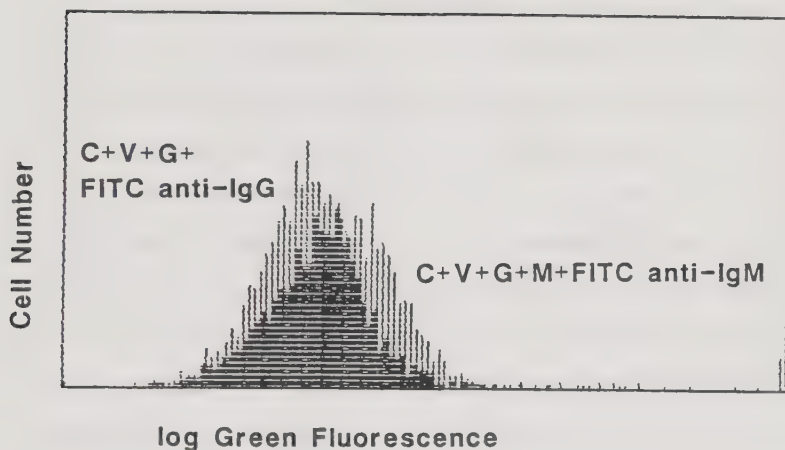


Figure 8. Relative fluorescence of a competition binding assay using two different neutralizing anti-BTV 17 monoclonal antibodies compared to cell-virus-complexes incubated with one monoclonal antibody. MDBK cells (C); infectious Bluetongue virus 17 (V); neutralizing monoclonal antibody 83C8-B1C, isotype G (G); neutralizing monoclonal antibody, 82F5-2B5, isotype M (M); antibody specific for mouse IgG conjugated with FITC (FITC anti-IgG); Antibody specific for mouse IgM conjugated with FITC (FITC anti-IgM).

DISCUSSION

The two BTV outer capsid proteins P2 and P5 are required for virus infectivity (66) and P2 is responsible for serotype-specificity and binding of neutralizing antibodies (24).

In our experiments, a panel of 13 neutralizing MABs was produced to BTV 17, and 7 of the antibodies precipitated P2. This indicates that all epitopes involved in neutralization which are recognized by these 7 MABs are found on P2. These findings support results from previously published experiments where immunoprecipitations with either BTV 17 neutralizing mouse ascites fluid, rabbit or sheep AB precipitated P2 (2, 24). The precipitation of P2 by these 7 MABs indicate that the neutralizing determinants of BTV 17 are either exclusively or predominantly located on P2. This is not surprising when the size of the two outer capsid proteins are compared. P2, a 98 KD protein, is 30 percent bigger than P5 (66 KD). Therefore, P2 might either predominate the neutralizing response, or alternatively overlap or cover P5 and thereby prevent recognition of the second outer capsid protein by the immune system.

Another factor which supports the view that P2 is the only protein with neutralizing epitopes lies in its function as viral attachment protein (VAP) (26). A well described mechanism of neutralization by antibody is the prevention of viral attachment to the cellular receptor site (41). Antibody directed against the VAP (here P2) can prevent viral attachment

to the cell receptor and thus can cause neutralization. Therefore, it is reasonable that the neutralizing epitopes are associated with P2.

Three explanations are conceivable for the failure of the other MABs to precipitate P2. First, these 6 clones may be very poor AB producers, and the AB concentration may not be high enough to precipitate the required amount of radiolabeled material to be visualized autoradiographically. Second, the affinity of these 6 monoclonals may be very low resulting in precipitation of insufficient amounts of radiolabeled material to be visualized by autoradiography in a reasonable length of time. Third, the neutralizing MABs may bind to a 3-dimensional conformation determinant on P2. It is well documented that the major outer capsid protein (P2) is very sensitive to degradation by salts and pH changes (26) and by hydrophobic solvents (66). At a pH of 5.0 only 0.2 M MgCl is required to solubilize P2, whereas at pH 8.0, 1.0 M MgCl is necessary to bring P2 in soluble fraction (26). It was also shown that virus infected cells treated with acetone or glutaraldehyde show altered binding to neutralizing ABs (2). This findings could be explained with conformational changes of the viral protein and its antigenic determinants.

The differences in the neutralizing activity can be explained in two ways: First, different MABs have different affinities to their attachment site on the virus. Antibody affinity is defined as the strength of a single antigen-

antibody bond which is produced by the summation of attractive and repulsive forces (53). Good fit between the antigenic determinant and the antibody binding site will result in high attraction and low repulsion, and create high affinity AB. Alternatively, poor fit between the antigenic determinant and the antibody binding site will result in high repulsive forces created by overlapping electron clouds, which will exceed any low attractive force. According to the Law of Mass Action, the equilibrium should theoretically be shifted to the antigen-antibody bound state by increasing the concentration of the antibody. However, it is conceivable that the equilibrium constant would be so small that the concentration of bound antigen-antibody could never reach the level necessary for visualizing by autoradiography within a reasonable period of time. The second explanation for the differences observed in the neutralizing activity could be found in the specificity of different MABs for different neutralizing epitopes. The mechanism of the neutralization of BTV at this point is not clear. It was demonstrated, however, that different antigenic determinants or epitopes are involved in neutralization (see results competition assay). Preliminary data in our laboratory suggest that the mechanism of neutralization employed in vitro by two MABs with strong neutralizing activity, 82F5-2B5 and 83C8-B1C, is by preventing the virus from binding to the cellular receptor site (data not shown). It is possible that blocking of epitopes on the viral attachment protein by other neutralizing ABs will result in a neutralization mechanisms which prevent viral internalization or viral uncoating. These

mechanisms might be less effective than prevention of attachment, which would result in the observed differences in neutralizing strength.

In a competition assay two neutralizing MABs with similar neutralizing activity were used to identify two unique epitopes on P2. The positive shift in fluorescence of the test cells, i.e. virus adsorbed cells incubated with saturating concentration of first MAB and subsequently incubated with a second competing MAB, indicates that these two monoclonal antibodies bind to different epitopes on P2. When an aliquot of the test cells was taken after the incubation of the first MAB and these cells were stained with FITC conjugated AB specific for the isotype of this first MAB. The analysis of this sample demonstrated that the first MAB was bound to the cell-virus complex. A second aliquot at the same step of the experiment was stained with FITC labeled AB specific for the isotype of the second MAB. The analysis of this sample demonstrated that the fluorescein label did not bind non-specifically to MABs.

One property of ABs which was not addressed so far is the intrinsic affinity. One might argue that the affinity of the second MAB could be higher than the affinity of the first MAB, and according to the Law of Mass action (see above) the equilibrium would shift to the binding of the second MAB. In the experiments, two MABs isotypes were used and they were incubated reciprocally, i.e. AB 1 competing for AB 2, and AB 2

competing for AB 1. Since similar results were obtained for both sequences of AB incubation, a difference in the intrinsic affinity of the MABs should not explain the results.

A different problem with this binding assay is steric hindrance. An AB binds generally to an epitope that spans over 5-7 amino acids (64). Therefore, one attached AB on the viral surface can block adjacent epitopes and prevent binding of other ABs. In using IgM MAB (82F5-2B5) as first and blocking AB and the IgG MAB (83C8-B1C) as second AB, it was demonstrated that the two epitopes which are bound by 82F5-2B5 and 83C8-B1C are spatially far apart. An alternative approach would use pepsin digestion fragments, called F_{ab} fragments, as first or blocking MAB. This would decrease the steric hindrance and allow better access to different viral determinants.

Alternatively, with 2 color analysis it is possible to demonstrate simultaneous binding of antibodies to a protein. Two different colored fluorescent tags, e.g. FITC and phycoerythrin, would be conjugated to antibodies with different isotypes. Again, the experiment would have to be performed where excess of AB is present. The comparison of MABs of the same isotype would be addressed by conjugating neutralizing MABs with fluorescent label and competing against non-labeled neutralizing MABs.

In summary, a panel of 13 monoclonal antibodies was produced which neutralizes BTV 17 in vitro. To characterize their biological function, their neutralizing activity was defined by virus neutralization test. Further, the viral protein involved in neutralization is viral protein P2, a 98 kilodalton protein. Finally, two different monoclonal antibodies were used to determine if more than one antigenic determinants or epitopes were involved in in vitro neutralization. The results suggest that at least 2 separate epitopes are present on P2 which are involved in in vitro neutralization.

DETECTION OF SHARED NEUTRALIZING EPITOPES BETWEEN DIFFERENT
BLUETONGUE VIRUS SEROTYPES

ABSTRACT

There is considerable evidence that both homologous (serotype-restricted) and cross-serotype-reactive protective immunity is important in the host defense against Bluetongue disease. The possible existence of shared or cross-reactive neutralizing epitopes among the five US Bluetongue virus (BTV) serotypes was thus investigated. Cross-neutralization assays with a panel of 13 neutralizing monoclonal antibodies produced to BTV serotype 17 was performed against the four BTV serotypes 2, 10, 11, and 13. There were four different patterns of neutralization observed: 1. one group of antibodies neutralized BTV 17 and 2; 2. one group neutralized BTV 17 and 11; 3. one group neutralized BTV 17, 2, and 11; and 4. one group neutralized BTV 17 alone. These results show that there are shared epitopes between BTV serotypes 2, 11, and 17 which are involved in neutralization. The importance of these results in context with heterotypic immunity and the development of vaccines are discussed.

DETECTION OF SHARED NEUTRALIZING EPITOPES BETWEEN DIFFERENT BLUETONGUE VIRUS SEROTYPES

Historically, the first attempts to control Bluetongue disease date back to the early years of this century. In 1906, Spreull introduced a method of immunizing sheep with hyperimmune serum of reconvalescent sheep in combination with virus (61 cited in 42, 62). These experiments were continued by Theiler over the following years by passing the virus in its natural host, the sheep. With continued passage, the virus lost its ability to cause fatal disease. This phenomenon led to extended laboratory and field trials and eventually to the release of the first BTV vaccine in 1907 (63). In 1929, du Toit (12) confirmed Theiler's method of immunization and introduced minor modifications to the standard procedure. Twenty years later, the multiplicity of BTV strains, today known as serotypes, was more carefully studied by Neitz (42). The plurality of the USA BTV serotypes was discovered shortly after the disease was described in 1952 (8). The two latest isolates were recorded in 1973 (3) and 1982 (9), respectively, being serotypes 17 and 2.

Neitz (42) was the first to demonstrate differences in virulence between BTV isolates, and showed that each strain or isolate of virus produces a solid immunity specifically against the same strain. Common immunity between strains, however, was characterized by great variability, a finding which supported the view of general serotype restricted immunity to BTV. More

recent studies by Jeggo and coworkers (28, 29) demonstrated that, in mice, the immune response to a second heterotypic BTV infection was enhanced when compared with the response to original infection with the same BTV serotype in an immunologically virgin individual. These findings indicate that infection with one serotype of BTV could influence the immune response to infection with a second serotype.

In general, antigen-antibody interactions are characterized by a high specificity of individual antibody molecules for their determinant on the antigen (53, 55). If two antigens have a similar or identical determinant, antibodies raised to one of the two antigens may bind to the other antigen and vice versa, i.e. cross-react. The phenomenon of cross-reactivity in vivo is called heterotypic immunity (1). Heterotypic immunity is a well characterized phenomenon in Influenza virus infection (47, 54, 68), and the mechanism for heterotypic immunity for this virus is the result of common or shared epitopes between Influenza virus hemagglutinin variants (1). In Bluetongue virus infections, the occurrence of heterotypic immunity has also been experimentally demonstrated. Jeggo (27) showed that sheep previously exposed to BTV serotypes 3 and 4 developed transient neutralizing antibodies to 18 other BTV serotypes. The control group which was exposed to BTV 3 only developed a small cross-reactive response to other serotypes. This response was of a much lower titer (<1:15 as compared to the test group with neutralizing antibody titers of >1:100) and reacted to only 3 other serotypes.

Beside the development of serum neutralizing ABs in the test animals, the dually infected sheep were also resistant to infection with a third BTV serotype, BTV 6, although it is unknown if protection was AB mediated.

One explanation for the heterotypic immune response to different BTV serotypes is shared or conserved epitopes among these different BTV serotypes. To test the hypothesis that there are shared neutralizing epitopes among the five US BTV serotypes a panel of 13 anti-BTV 17 neutralizing antibodies was used in an in vivo neutralization assay against 4 other BTV serotypes, BTV 2, 10, 11, and 13.

MATERIALS AND METHODS

Virus. Bluetongue virus serotypes 2, 10, 11, and 13 were kindly provided by Drs. Jochim and Luedke, USDA. BTV serotype 2 was obtained in sheep blood. To adapt the virus to tissue culture cells, several blind passages in Vero M cells were performed before infectious virus was growing in and could be harvested from MDBK cells. Subsequently, all virus serotypes were plaque cloned three times, and this cloned virus was expanded for use in the following experiments. Each serotype specificity was assured in a virus neutralization assay using known monospecific typing sera (also provided by Drs. Jochim and Luedke, USDA).

Cross-Neutralization Assay. Plaque cloned BTV serotypes 2, 10, 11, and 13 were used in cross-neutralization assays. Fifty μ l of anti-BTV 17 neutralizing MABs hybridoma culture supernatant were incubated with 5×10^3 or 5×10^2 PFUs of the BTV serotypes 2, 10, 11, and 13 according to the standard protocol for virus neutralization and hybridoma screening (see "Production and Characterization of Neutralizing Monoclonal Antibodies against Bluetongue Virus 17"). After 1 hour incubation at 4°C the mixture was incubated on MDBK cell monolayers in 96-well-tissue-culture plates and covered with minimum essential media (MEM) (Gibco Laboratories, Grand Island, NY) containing 0.5 percent agarose (Miles type 1S, Miles Scientific Laboratories, Naperville, IL). After 48 hours incubation at 37°C cells were stained with crystal violet for

several hours, the agar overlay was removed and the monolayer thoroughly washed under running water. Individual wells were evaluated for plaques by light microscopy to insure the correct evaluation of differences among true plaque formation, monolayer lesions, and staining heterogeneity. These assays used two different virus dilutions to detect lower cross-neutralizing activities. The evaluation of the assay was as follows: Complete protection of 5×10^3 PFUs BTV of any serotype by 50 μ l of culture supernatant was called strong cross-neutralization. A plaque reduction which was greater than one fourth as compared to the well with the negative control was called weak cross-neutralization. Clones which showed strong or weak cross-neutralization in both virus dilutions, i.e. 5×10^3 and 5×10^2 PFUs, were called positive cross-neutralizers. Clones which showed less than one fourth plaque reduction in either or both virus dilutions were called negative cross-neutralizers.

RESULTS

Cross-Neutralizing Activity of Bluetongue-Virus-17-Specific Monoclonal Antibodies with Other Bluetongue Virus Serotypes

The neutralizing activity of anti-BTV 17 specific MABs were assessed in cross-neutralization assays with 4 other serotypes. Results are summarized in table 3. No cross-neutralizing activity was observed with either BTV serotype 10 or 13 (data not shown). Four different patterns of reactivity were observed in the cross-neutralizing assays with BTV serotypes 2 and 11: A group of 8 MABs neutralized BTV serotype 17 alone; one MAB (94B5-10A) neutralized BTV serotype 2 in addition to serotype 17; two MABs (92F8-8N and 94G10-8A) neutralized BTV serotypes 17 and 11, but not type 2; and finally two other MABs (82F5-2B5 and 83C8-B1C) neutralized BTV serotypes 2, 11, and 17. The neutralizing activities are presented in table 4.

BLUETONGUE VIRUS SEROTYPE SPECIFICITY

17	17, 2	17, 11	17, 2, 11
83B2-IIA	94B5-10A	92F8-8N	82F5-2B5
83B8-3A		94G10-8A	83C8-B1C
91E2-2L8			
92B4-B2			
92E5-2A			
93H5-1B			
93E8-8E			
94G9-4B16			

Table 3. Cross-neutralizing activity of anti-BTV 17 neutralizing monoclonal antibodies against BTV serotypes 2, 11, and 17.

MONOCLONAL ANTIBODY	CROSS-NEUTRALIZING ACTIVITY AGAINST	
	BTV 11	BTV 2
82F5-2B5	weak	weak
83B2-3A	-	-
83B2-IIA	-	-
83C8-B1C	weak	weak
91E2-2I8	-	-
92B4-B2	-	-
92E5-2A	-	-
92F8-8N	weak	-
93H5-1B	-	-
93E8-8E	-	-
94B5-10A	-	weak
94G9-4B16	-	-
94G10-8A	weak	-

Table 4. Cross-neutralizing activity of anti-BTV 17 neutralizing monoclonal antibodies against BTV serotypes 2 and 11. Plaque reduction greater than one fourth = weak neutralization; Plaque reduction less than one fourth = - neutralization.

DISCUSSION

The variability among the BTV serotypes (51) and within each serotype (i.e. variability among different isolates of the same serotype) (58), can be explained by two observations:

1) As a member of the family Reoviridae, BTV has a segmented RNA genome. Generally, RNA viruses have a spontaneous mutation rate of 10^{-3} to 10^{-4} mutations per inoculated virion (20, 53). This high incidence of mutations is due to the low fidelity of RNA replication, i.e. lack of proofreading activity of the RNA replicating enzymes. 2) The segmented type of genome of this virus allows BTV to reassort its genome. Reassortment is defined as the random combination of gene segments from parental viruses in the progeny virus (50). More recently, in vivo reassortment of BTV in the bovine was demonstrated (43, and personal communication with Dr. Oberst, University of Davis, CA). Viremic cattle infected with BTV serotypes 11 and 17, or with two electrophoretically different isolates of the same serotype showed progeny virus which was identified as reassortment of the inoculated viruses. Progeny reassortment virus was isolated in a majority (89 percent) of the animals infected with 2 different serotypes. Mixed strain or isolate infection resulted in a low percentage of reassortments which was only observed during the peak of the viremia. These findings confirm that the previously stated general rules on reassortment of segmented RNA viruses are true for Bluetongue virus.

Based on fingerprint analysis, Rao et al. (52) found a close genetic relationship between BTV serotypes 11 and 17, and less homology when either serotype was compared to serotypes 10 or 13, respectively. These authors hypothesised that BTV serotype 17 may have evolved from serotype 11 by genetic reassortment over a period of time. An alternative explanation would be a common ancestry for both these serotypes, i.e. there is no direct parent-progeny relationship but rather both serotypes might have evolved from a common virus. This second explanation seems less likely because one might expect to find the ancestor in the USA. If serotypes 11 and 17 are related then it might be expected that they share common determinants.

In fact, within the panel of 13 anti-BTV 17 neutralizing MABs we detected 5 MABs which displayed in vitro neutralizing activity to serotypes other than BTV serotype 17. Cross-neutralization assays showed 4 pattern of reactivity: One group of MABs neutralized BTV 2 in addition to BTV 17; a second group neutralized BTV 17 and 11, but not BTV 2; a third group neutralized BTV 2, 11, and 17; and a fourth group of MABs neutralized BTV 17 alone. These results indicate that antigenic determinants are shared between BTV serotypes 2, 11, and 17, but with this panel no shared antigenic determinants could be determined which are shared between BTV serotype 17 and either serotypes 10 or 13. These findings are in agreement with the aforementioned work by Rao (52) where more genetic diversity between serotypes 10 and 13 compared with serotype 17 were observed than between serotypes 11 and 17.

It is interesting that we determined additional shared neutralizing epitopes between BTV serotype 2 and 17. BTV 2 is the most recently described BTV serotype in the USA (first isolated in 1982) and was not present in the USA at the time of the publication of Rao's experiments. The same situation as for BTV 11 and 17 might be true for either BTV 11 and 2, or BTV 17 and 2. Antigenic changes in the parental virus might have led to a new serotype, BTV 2. BTV 17 was isolated and described 7 years after BTV 11; BTV 2 was first described 9 years after BTV 17.

This knowledge on the relationship between serotypes of BTV and the sharing of epitopes strengthens the hypothesis that a possible mechanism for heterotypic immunity is the recognition of shared or conserved epitopes. Additionally, these findings are in agreement with documentations about shared epitopes in rotaviruses (7, 14, 70), another member of the family reoviridae.

Another unusual finding should be mentioned. Four out of the 5 cross-neutralizing monoclonals belong to the IgM class. The reason why IgM clones seem to have better cross-reactivity is unknown. If IgM clones would express non-specific cross-reactive activity it would be expected that all MABs of the M isotype should exhibit this same non-specific neutralizing activity on all other BTV serotypes. This, however, was not the case. Additionally, the negative control in all the assays was an anti-Toxocara ascaris IgM MAB. This IgM monoclonal failed also to exhibit non-specific activity to any BTV serotype.

It is clear that this data indicates only the beginning of a project to determine the relationship between serotypes and the sharing of epitopes. The knowledge of these relationships, however, will be very helpful in the development of new vaccines. Vaccines composed of several attenuated BTV serotypes could be replaced by a subunit vaccine representing shared epitopes. This approach would have several advantages:

- 1) If the epitope is selected for induction of strong neutralizing ABs, the expected immune response would exceed a whole virus vaccine;
- 2) a subunit vaccine would eliminate the concern of recombination wild type virus with vaccine virus;
- 3) animals inoculated with a subunit vaccine would easily be distinguishable from animals which were naturally infected; and
- 4) this vaccine should be safe in pregnant animals.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

To better define the role and the relationship of antigenic determinants on the viral proteins of different BTV serotypes in protective immunity, we developed a panel of neutralizing monoclonal antibodies to BTV serotype 17. The virus was mouse adapted by growth in neonatal mouse brain and subsequently used as immunogen in adult Balb/c mice. Thirteen clones from two different fusions were selected by in vitro neutralization assays for the following experiments.

To determine which viral protein bound by our neutralizing MABs, radioimmunoprecipitations with virally infected ^{35}S -methionine labeled cell lysate were performed and analysed by 10 percent SDS-polyacrylamide-gel-electrophoresis. The results showed that all tested MABs precipitated a 98 kilodalton viral protein, BTV-protein P2, as determined by comparison with bands obtained by precipitations with monospecific anti-BTV 17 typing serum and monoclonal antibody against BTV 17 proteins P2, P7, and P9, respectively.

To determine whether all MABs were directed to the same epitope on P2, competition binding assays were analysed by flow cytometry. Semipurified BTV 17 was adsorbed to susceptible bovine cells (MDBK cells) using 100 infectious virus particles per cell. In the first step, a neutralizing MAB of one isotype (an IgM) was adsorbed to the virus on the cell surface. In the second step, a second neutralizing MAB of a different isotype (an IgG) was incubated with the complex and allowed to bind.

For the analysis, the complex was stained with a fluorescein isothiocyanate (FITC) labeled AB specific for the isotype of the second MAB. If both monoclonal antibodies bound the same or a spacially close epitope, the second MABs could not bind, and no positive fluorescence would be detected. Alternatively, if the second MAB (the IgG) bound a spacially separate and different epitope from the one recognized by the IgM, and this IgM MAB was adsorbed in saturating amount, with the appropriate label (a FITC conjugated anti-IgG heavy chain specific antibody) one would see positive fluorescence. The analysis showed positive fluorescence for the assay with the two competing MABs. This was determined by comparison with a negative control (virus adsorbed on cells, incubated only with neutralizing IgM, and stained with FITC conjugated anti-IgG) and with a positive control (virus adsorbed on cells, incubated with neutralizing IgM, and stained with FITC conjugated anti-IgM). The same results were obtained when the IgG monoclonal was used as first or blocking AB and the IgM monoclonal as second AB. These results are good evidence that these two different isotypes of neutralizing MABs bind to different epitopes.

To test the hypothesis that there are shared neutralizing epitopes between different BTV serotypes, these 13 MABs were used in cross-neutralization assays. Standard virus neutralization assays with the panel of anti-BTV 17 neutralizing MABs and the other four US BTV serotypes (serotypes 2, 10, 11, and 13) revealed 4 different patterns of

reactivity: One group of MABs neutralized BTV 17 alone; a second group neutralized BTV 2 in addition to BTV 17; a third group neutralized BTV 17 and 11, but not BTV 2; and a fourth group neutralized BTV 2, 11, and 17. No cross-neutralization was detected with either BTV 10 or 13. These results suggest that there are shared or conserved epitopes among BTV serotypes. These findings are important for different aspects of BT disease: 1) The vaccine production, 2) the understanding of protective immune mechanisms, 3) the explanation for heterotypic immunity.

Currently available BTV vaccines are attenuated or modified live virus preparations which have the potential of reassortment with wild type, reversion to virulence, and induction of disease in fetuses and adults. A promising way to avoid these problems is the use of either a subunit vaccine or an anti-idiotypic vaccine. In both cases, it is necessary to characterize the appropriate viral protein and epitopes responsible for eliciting the protective immune response.

In a subunit vaccine, only one or a few proteins, or maybe only one peptide representing the critical epitope, replace the infectious agent in the inoculum. In BTV, this approach was successful in the mouse model (44), where dissociated outer capsid proteins induced ABs which bound to intact BTV particles adsorbed on sheep erythrocytes. In another laboratory (26), isolated and solubilized viral protein P2 from purified Bluetongue virus stimulated the production of neutralizing ABs in rabbits and sheep.

The problem with this approach is the nature of P2 and its determinants. P2 and its neutralizing epitopes are very labile as shown by its denaturation in different solvents (2, 26, 66). Additionally, the serial production of subunit and peptide for vaccines is complicated by the existence of discontinuous or conformational epitopes (5, 6, 30, 59). Conformational epitopes result from the tertiary structure of the amino acid sequence. Loops of crossing over amino acid sequences form epitopes which function as antigenic sites. It is not clear at present, whether the neutralizing epitopes on P2 are of primary amino acid sequence or whether they are conformational.

Despite these unsolved problems, the importance and significance of the presented data is indispensable. Shared neutralizing epitopes between different BTV serotypes provide an explanation for the phenomenon of heterotypic immunity and thereby provide information for a reassessment of BTV vaccines.

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